

**Fig. 5.** Effects of unloading on trabecular and cortical bone in mice with targeted deletion of  $ER\alpha$  in osteocytes. (A and C) Representative  $\mu$ CT views. (B) 3D measurements of femoral distal trabecular area and (D) 3D measurements of femoral diaphyses from  $ER\alpha^{flox/flox}$  and  $ER\alpha^{\Delta Ocy/\Delta Ocy}$  mice subjected or not subjected to tail suspension. Data are represented as mean  $\pm$  SEM (n = 6). \* indicates  $p < 0.05$ .

knockout mice by Maatta et al. and Almeida et al., and in previous reports regarding osteoclast-specific ER $\alpha$  knockout mice [8,9]. As would be predicted, androgen receptor knockout mice (ARKO), including both systemic ARKO [51] and osteocyte conditional ARKO [52], exhibited bone loss in male mice. These gender-specific phenotypes are probably caused by differences in concentration of circulating sex steroids, estrogens and androgens. In contrast to these studies and our present study, a recent report showed that mice lacking ER $\alpha$  using the same Dmp1-Cre mouse exhibited trabecular bone loss only in male mice, but not in female mice [13]. In this report, Windahl et al. proposed that the physiological trabecular bone-sparing effect of estrogen is mediated via ER $\alpha$  in osteocytes in males, but also via ER $\alpha$  in osteoclasts in females [13]. At present, it is difficult to provide a convincing explanation to describe the discrepancies between our current study and this report [13]. However, one possible reason may be differences in the genetic background of the mouse strain of the ER $\alpha$ -floxed mice since the Dmp1-Cre mice were identical. The ER $\alpha$ -floxed mice used in our study have been registered as *Esr1<sup>tm1Mma</sup>* and originated from 129S2/SvPas mixed background, and published in 2000 [5], then backcrossed with C57BL6 line for more than 10 times. On the other hand, the ER $\alpha$ -floxed mice used in the study by Windahl et al. have been registered as *Esr1<sup>tm1Gust</sup>* and originated from 129X1/Svj mixed background, and published in 2012 [53]. These differences might be responsible for the discrepancies between the two studies. Regardless, the results of these two studies suggest that osteocytic ER $\alpha$  may have a role in maintenance of trabecular bone homeostasis regardless of gender.

To investigate the possible molecular basis underlying ER $\alpha$  function in osteocytes, we performed an osteocyte isolation technique using FACS analysis of Dmp1-GFP positive cells from conditional null mice and their controls. The results obtained from the Functional Annotation Clustering of differentially expressed genes suggested that osteocytic ER $\alpha$  might regulate transcription of the genes related to secretory proteins, which may regulate osteoblastic bone formation and contribute to maintenance of bone homeostasis. In fact, *Sostdc1*, an antagonist of the Wnt signaling [45,54], was elevated as a downstream gene of osteocytic ER $\alpha$ . *Sostdc1* is a gene also called *Wise* or *Ectodm* whose domain is similar to *Sost* (Sclerostin). *Sost* and *Sostdc1* bind to Wnt co-receptors called *Lrps* and regulate the Wnt/ $\beta$ -catenin pathway negatively [55]. Wnt signal proteins are reported to modulate bone mass *in vivo* by acting directly on mesenchymal stem cells [56–59]. Genes involved in the Wnt signaling are known to regulate the cell proliferation, differentiation, and apoptosis of osteoblasts [60]. Interaction between  $\beta$ -catenin and ER $\alpha$  has been previously reported [61] and the expressions of some Wnt family genes are important for responding to mechanical stress and are reportedly regulated by ER $\alpha$  [32]. Conventional *Sostdc1* KO mice are reported to exhibit abnormal tooth development, which has similar characteristics as bone [45,54]. Also, it has been reported that estradiol regulates mRNA levels of *Sostdc1* in U2OS cells [62]. In addition, a meta-analysis of BMD in a female Chinese population revealed that a mutation in the *Sostdc1* coding region was correlated with BMD, suggesting that *Sostdc1* might play a role in homeostasis of bone metabolism [46].

Also, *Midkine*, *Mdk*, was elevated as a downstream molecule of ER $\alpha$  in mice with this targeted deletion. *Mdk* is a member of a family of heparin-binding growth factors known primarily for their effects on neural cells [63]. *Mdk* expression is reported to increase during the course of primary osteoblast differentiation. *Mdk* has been shown to bind to a complex of protein tyrosine phosphatase zeta (*Ptprx*), low-density lipoprotein receptor-related protein-6 (*Lrp6*), and exert negative effects on Wnt signaling [64]. Conventional *Mdk* null mice exhibit increased bone formation, suggesting *Mdk* is a negative regulator of osteoblastic bone formation. Furthermore, *Mdk* KO mice are resistant to OVX-induced bone loss and sensitive to mechanical loading induced cortical bone increase [44]. In addition, the expression of ALP and the induction of canonical Wnt signaling in MC3T3E1, an osteoblastic cell line, were inhibited by *Mdk* treatments [64]. These reports and the results

from our current study suggest that *Sostdc1* and *Mdk* might be responsible for a component of estrogen's osteoprotective actions.

However, questions remain regarding how ER $\alpha$  negatively regulates the transcription of these genes because there are no reports of a negative transcriptional regulation of the estrogen receptor response element (negative ERE), although details of a negative glucocorticoid receptor response element (nGRE) have been reported [65]. Alternatively, it is possible that the expression of these factors might be regulated by an ER $\alpha$ -dependent miRNA. The precise molecular basis of transcriptional regulation or mRNA stabilization of these genes must be clarified in future studies. Neutralizing or deletion studies of these two proteins in this mouse model could provide possible answers for these questions.

In conclusion, osteocytic ER $\alpha$  might play a role in estrogen's osteoprotective action by controlling the expression of Wnt antagonists, which regulate osteoblastic bone formation in trabecular bone.

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### Conflict of interest

All authors state that they have no conflicts of interest.

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### References

- [1] Nelson HD. Menopause. *Lancet* 2008;371:760–70.
- [2] Imai Y, Kondoh S, Kouzmenko A, Kato S. Minireview: osteoprotective action of estrogens is mediated by osteoclastic estrogen receptor- $\alpha$ . *Mol Endocrinol* 2010;24:877–85.
- [3] Sun L, Peng Y, Sharrow AC, Iqbal J, Zhang Z, Papachristou DJ, et al. FSH directly regulates bone mass. *Cell* 2006;125:247–60.
- [4] Pacifici R. Estrogen deficiency, T cells and bone loss. *Cell Immunol* 2008;252:68–80.
- [5] Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P, Mark M. Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* 2000;127:4277–91.
- [6] Windahl SH, Andersson G, Gustafsson JA. Elucidation of estrogen receptor function in bone with the use of mouse models. *Trends Endocrinol Metab* 2002;13:195–200.
- [7] Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, et al. Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-beta in bone remodeling in females but not in males. *Bone* 2002;30:18–25.
- [8] Nakamura T, Imai Y, Matsumoto T, Sato S, Takeuchi K, Igarashi K, et al. Estrogen prevents bone loss via estrogen receptor alpha and induction of Fas ligand in osteoclasts. *Cell* 2007;130:811–23.
- [9] Martin-Millan M, Almeida M, Ambrogini E, Han L, Zhao H, Weinstein RS, et al. The estrogen receptor- $\alpha$  in osteoclasts mediates the protective effects of estrogens on cancellous but not cortical bone. *Mol Endocrinol* 2010;24:323–34.
- [10] Tomkinson A, Reeve J, Shaw RW, Noble BS. The death of osteocytes via apoptosis accompanies estrogen withdrawal in human bone. *J Clin Endocrinol Metab* 1997;82:3128–35.
- [11] Lee K, Jessop H, Suswillo R, Zaman G, Lanyon L. Endocrinology: bone adaptation requires oestrogen receptor- $\alpha$ . *Nature* 2003;424:389.
- [12] Maatta JA, Buki KG, Gu G, Alanne MH, Vaaranenmi J, Liljenback H, et al. Inactivation of estrogen receptor alpha in bone-forming cells induces bone loss in female mice. *FASEB J* 2012;27:478–88.
- [13] Windahl SH, Borjesson AE, Farman HH, Engdahl C, Moverare-Skrtic S, Sjogren K, et al. Estrogen receptor- $\alpha$  in osteocytes is important for trabecular bone formation in male mice. *Proc Natl Acad Sci U S A* 2013;110:2294–9.
- [14] Almeida M, Iyer S, Martin-Millan M, Bartell SM, Han L, Ambrogini E, et al. Estrogen receptor- $\alpha$  signaling in osteoblast progenitors stimulates cortical bone accrual. *J Clin Invest* 2013;123:394–404.
- [15] Melville KM, Kelly NH, Khan SA, Schimenti JC, Ross FP, Main RP, et al. Female mice lacking estrogen receptor- $\alpha$  in osteoblasts have compromised bone mass and strength. *J Bone Miner Res* 2013 (in press) (Epub ahead of print).
- [16] Burger EH, Klein-Nulend J. Mechanotransduction in bone—role of the lacuno-canalicular network. *FASEB J* 1999(13 Suppl.):S101–12.



- [17] Robling AG, Niziolek PJ, Baldrige LA, Condon KW, Allen MR, Alam I, et al. Mechanical stimulation of bone in vivo reduces osteocyte expression of Sost/sclerostin. *J Biol Chem* 2008;283:5866–75.
- [18] Skerry TM, Bitensky L, Chayen J, Lanyon LE. Early strain-related changes in enzyme activity in osteocytes following bone loading in vivo. *J Bone Miner Res* 1989;4:783–8.
- [19] Gluhak-Heinrich J, Ye L, Bonewald LF, Feng JQ, MacDougall M, Harris SE, et al. Mechanical loading stimulates dentin matrix protein 1 (DMP1) expression in osteocytes in vivo. *J Bone Miner Res* 2003;18:807–17.
- [20] Gluhak-Heinrich J, Pavlin D, Yang W, MacDougall M, Harris SE. MEPE expression in osteocytes during orthodontic tooth movement. *Arch Oral Biol* 2007;52:684–90.
- [21] Thompson DL, Sabbagh Y, Tenenhouse HS, Roche PC, Drezner MK, Salisbury JL, et al. Ontogeny of PheX/PHEX protein expression in mouse embryo and subcellular localization in osteoblasts. *J Bone Miner Res* 2002;17:311–20.
- [22] Nampai A, Hashimoto J, Hayashida K, Tsuboi H, Shi K, Tsuji I, et al. Matrix extracellular phosphoglycoprotein (MEPE) is highly expressed in osteocytes in human bone. *J Bone Miner Metab* 2004;22:176–84.
- [23] Liu S, Zhou J, Tang W, Jiang X, Rowe DW, Quarles LD. Pathogenic role of Fgf23 in Hyp mice. *Am J Physiol Endocrinol Metab* 2006;291:E38–49.
- [24] Feng JQ, Ward LM, Liu S, Lu Y, Xie Y, Yuan B, et al. Loss of DMP1 causes rickets and osteomalacia and identifies a role for osteocytes in mineral metabolism. *Nat Genet* 2006;38:1310–5.
- [25] Bonewald LF. Osteocytes as dynamic multifunctional cells. *Ann N Y Acad Sci* 2007;1116:281–90.
- [26] Bergmann P, Body JJ, Boonen S, Boutsens Y, Devogelaer JP, Goemaere S, et al. Loading and skeletal development and maintenance. *J Osteoporos* 2010;2011:786752.
- [27] Lloyd SA, Bandstra ER, Willey JS, Riffle SE, Tirado-Lee L, Nelson GA, et al. Effect of proton irradiation followed by hindlimb unloading on bone in mature mice: a model of long-duration spaceflight. *Bone* 2012;51:756–64.
- [28] Lee KC, Maxwell A, Lanyon LE. Validation of a technique for studying functional adaptation of the mouse ulna in response to mechanical loading. *Bone* 2002;31:407–12.
- [29] Bonewald LF, Johnson ML. Osteocytes, mechanosensing and Wnt signaling. *Bone* 2008;42:606–15.
- [30] Robinson JA, Chatterjee-Kishore M, Yaworsky PJ, Cullen DM, Zhao W, Li C, et al. Wnt/beta-catenin signaling is a normal physiological response to mechanical loading in bone. *J Biol Chem* 2006;281:31720–8.
- [31] Javaheri B, Stern A, Lara N, Dallas M, Zhao H, Liu Y, et al. Deletion of a single beta-catenin allele in osteocytes abolishes the bone anabolic response to loading. *J Bone Miner Res* 2013 (in press) (Epub ahead of print).
- [32] Armstrong VJ, Muzylak M, Suinters A, Zaman G, Saxon LK, Price JS, et al. Wnt/beta-catenin signaling is a component of osteoblastic bone cell early responses to load-bearing and requires estrogen receptor alpha. *J Biol Chem* 2007;282:20715–27.
- [33] Lu Y, Xie Y, Zhang S, Dusevich V, Bonewald LF, Feng JQ. DMP1-targeted Cre expression in odontoblasts and osteocytes. *J Dent Res* 2007;86:320–5.
- [34] Kalajzic I, Braut A, Guo D, Jiang X, Kronenberg MS, Mina M, et al. Dentin matrix protein 1 expression during osteoblastic differentiation, generation of an osteocyte GFP-transgene. *Bone* 2004;35:74–82.
- [35] Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, Muller R. Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *J Bone Miner Res* 2010;25:1468–86.
- [36] Sato S, Hanada R, Kimura A, Abe T, Matsumoto T, Iwasaki M, et al. Central control of bone remodeling by neuregulin. *U Nat Med* 2007;13:1234–40.
- [37] Parfitt AM, Drezner MK, Glorieux FH, Kanis JA, Malluche H, Meunier PJ, et al. Bone histomorphometry: standardization of nomenclature, symbols, and units. Report of the ASBMR Histomorphometry Nomenclature Committee. *J Bone Miner Res* 1987;2:595–610.
- [38] Paic F, Igwe JC, Nori R, Kronenberg MS, Franceschetti T, Harrington P, et al. Identification of differentially expressed genes between osteoblasts and osteocytes. *Bone* 2009;45:682–92.
- [39] Huang da W, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. *Nat Protoc* 2009;4:44–57.
- [40] McCarthy JJ, Fox AM, Tsika GL, Gao L, Tsika RW. Beta-MHC transgene expression in suspended and mechanically overloaded/suspended soleus muscle of transgenic mice. *Am J Physiol* 1997;272:R1552–61.
- [41] Tsika G, Ji J, Tsika R. Sp3 proteins negatively regulate beta myosin heavy chain gene expression during skeletal muscle inactivity. *Mol Cell Biol* 2004;24:10777–91.
- [42] Xiong J, Onal M, Jilka RL, Weinstein RS, Manolagas SC, O'Brien CA. Matrix-embedded cells control osteoclast formation. *Nat Med* 2011;17:1235–41.
- [43] Heine PA, Taylor JA, Iwamoto GA, Lubahn DB, Cooke PS. Increased adipose tissue in male and female estrogen receptor-alpha knockout mice. *Proc Natl Acad Sci U S A* 2000;97:12729–34.
- [44] Neunaber C, Catala-Lehnen P, Beil FT, Marshall RP, Kanbach V, Baranowsky A, et al. Increased trabecular bone formation in mice lacking the growth factor midkine. *J Bone Miner Res* 2010;25:1724–35.
- [45] Ahn Y, Sanderson BW, Klein OD, Krumlauf R. Inhibition of Wnt signaling by Wise (Sostdc1) and negative feedback from Shh controls tooth number and patterning. *Development* 2010;137:3221–31.
- [46] He JW, Yue H, Hu WW, Hu YQ, Zhang ZL. Contribution of the sclerostin domain-containing protein 1 (SOSTDC1) gene to normal variation of peak bone mineral density in Chinese women and men. *J Bone Miner Metab* 2011;29:571–81.
- [47] Tou JC, Foley A, Yuan YV, Arnaud S, Wade CE, Brown M. The effect of ovariectomy combined with hindlimb unloading and reloading on the long bones of mature Sprague-Dawley rats. *Menopause* 2008;15:494–502.
- [48] Almeida M, Martín-Millán M, Ambrogini E, Bradsher 3rd R, Han L, Chen XD, et al. Estrogens attenuate oxidative stress and the differentiation and apoptosis of osteoblasts by DNA-binding-independent actions of the ERalpha. *J Bone Miner Res* 2010;25:769–81.
- [49] Tomkinson A, Gevers EF, Wit JM, Reeve J, Noble BS. The role of estrogen in the control of rat osteocyte apoptosis. *J Bone Miner Res* 1998;13:1243–50.
- [50] Krum SA, Miranda-Carboni GA, Hauschka PV, Carroll JS, Lane TF, Freedman LP, et al. Estrogen protects bone by inducing Fas ligand in osteoblasts to regulate osteoclast survival. *EMBO J* 2008;27:535–45.
- [51] Kawano H, Sato T, Yamada T, Matsumoto T, Sekine K, Watanabe T, et al. Suppressive function of androgen receptor in bone resorption. *Proc Natl Acad Sci U S A* 2003;100:9416–21.
- [52] Sinnesael M, Claessens F, Laurent M, Dubois V, Boonen S, Deboel L, et al. Androgen receptor (AR) in osteocytes is important for the maintenance of male skeletal integrity: evidence from targeted AR disruption in mouse osteocytes. *J Bone Miner Res* 2012;27:2535–43.
- [53] Antonson P, Omoto Y, Humire P, Gustafsson JA. Generation of ERalpha-floxed and knockout mice using the Cre/LoxP system. *Biochem Biophys Res Commun* 2012;424:710–6.
- [54] Cho SW, Kwak S, Woolley TE, Lee MJ, Kim EJ, Baker RE, et al. Interactions between Shh, Sostdc1 and Wnt signaling and a new feedback loop for spatial patterning of the teeth. *Development* 2011;138:1807–16.
- [55] Itasaki N, Jones CM, Mercurio S, Rowe A, Domingos PM, Smith JC, et al. Wise, a context-dependent activator and inhibitor of Wnt signalling. *Development* 2003;130:4295–305.
- [56] Liu G, Vijayakumar S, Grumolato L, Arroyave R, Qiao H, Akiri G, et al. Canonical Wnts function as potent regulators of osteogenesis by human mesenchymal stem cells. *J Cell Biol* 2009;185:67–75.
- [57] Takada I, Kouzmenko AP, Kato S. Wnt and PPARgamma signaling in osteoblastogenesis and adipogenesis. *Nat Rev Rheumatol* 2009;5:442–7.
- [58] Augello A, De Bari C. The regulation of differentiation in mesenchymal stem cells. *Hum Gene Ther* 2010;21:1226–38.
- [59] Baron R, Kneissel M. WNT signaling in bone homeostasis and disease: from human mutations to treatments. *Nat Med* 2013;19:179–92.
- [60] Westendorf JJ, Kahler RA, Schroeder TM. Wnt signaling in osteoblasts and bone diseases. *Gene* 2004;341:19–39.
- [61] Kouzmenko AP, Takeyama K, Ito S, Furutani T, Sawatsubashi S, Maki A, et al. Wnt/beta-catenin and estrogen signaling converge in vivo. *J Biol Chem* 2004;279:40255–8.
- [62] Tee MK, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy RJ, et al. Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. *Mol Biol Cell* 2004;15:1262–72.
- [63] Nakamura E, Kadomatsu K, Yuasa S, Muramatsu H, Mamiya T, Nabeshima T, et al. Disruption of the midkine gene (Mdk) resulted in altered expression of a calcium binding protein in the hippocampus of infant mice and their abnormal behaviour. *Genes Cells* 1998;3:811–22.
- [64] Liedert A, Mattausch L, Rontgen V, Blakytyn R, Vogeel D, Pahl M, et al. Midkine-deficiency increases the anabolic response of cortical bone to mechanical loading. *Bone* 2011;48:945–51.
- [65] Surjit M, Ganti KP, Mukherji A, Ye T, Hua G, Metzger D, et al. Widespread negative response elements mediate direct repression by agonist-liganded glucocorticoid receptor. *Cell* 2011;145:224–41.



# Hox5 interacts with *Plzf* to restrict *Shh* expression in the developing forelimb

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To date, only the five most posterior groups of *Hox* genes, *Hox9–Hox13*, have demonstrated loss-of-function roles in limb patterning. Individual paralog groups control proximodistal patterning of the limb skeletal elements. *Hox9* genes also initiate the onset of *Hand2* expression in the posterior forelimb compartment, and collectively, the posterior *HoxA/D* genes maintain posterior *Sonic Hedgehog* (*Shh*) expression. Here we show that an anterior *Hox* paralog group, *Hox5*, is required for forelimb anterior patterning. Deletion of all three *Hox5* genes (*Hoxa5*, *Hoxb5*, and *Hoxc5*) leads to anterior forelimb defects resulting from derepression of *Shh* expression. The phenotype requires the loss of all three *Hox5* genes, demonstrating the high level of redundancy in this *Hox* paralogous group. Further analyses reveal that *Hox5* interacts with *promyelocytic leukemia zinc finger* biochemically and genetically to restrict *Shh* expression. These findings, along with previous reports showing that point mutations in the *Shh* limb enhancer lead to similar anterior limb defects, highlight the importance of *Shh* repression for proper patterning of the vertebrate limb.

limb development | organogenesis | anteroposterior limb patterning | gene interactions | mouse developmental genetics

Limb buds initially emerge as small bulges protruding from the embryonic lateral plate mesenchyme, and development proceeds along three axes: dorsoventral (DV), proximodistal (PD), and anteroposterior (AP) (1). Numerous factors involved in the establishment of these three axes have been defined; for example, DV patterning depends on the antagonism between *Wnt7a* from the dorsal ectoderm and bone morphogenetic protein genes (*BMPs*) and *Engrailed1* (*EN1*) from the ventral ectoderm, growth along the PD axis is regulated mainly by fibroblast growth factor genes (*Fgfs*) secreted from the apical ectodermal ridge (AER), and establishment of the AP axis requires signaling from a region of the posterior limb bud termed the zone of polarizing activity (ZPA). *Sonic Hedgehog* (*Shh*) is the morphogen secreted from this region (2), and loss of *Shh* function results in the absence of posterior limb elements (3, 4). Previous research has identified a limb-specific enhancer located in the fifth intron of *limb region 1 protein homolog* gene approximately 1 Mb from the *Shh* coding sequence, designated the ZPA regulatory sequence (ZRS) (5). Deletion of this enhancer leads to defects similar to *Shh* loss-of-function mutants (6–8).

*Hox* genes also have been shown to play pivotal roles in limb PD patterning of the limb skeletal elements. The *HoxA* and *HoxD* genes from groups 9–13 impact forelimb development along the PD axis (9–14). *Hoxa9/d9* and *Hox10* paralogs specify stylopod patterning (humerus and femur) (10, 14, 15). Loss of function of *Hoxa11* and *Hoxd11* results in dramatic mispatterning of the zeugopod (radius/ulna and tibia/fibula) (9, 14). Loss of autopod elements (i.e., handplate and footplate) in *Hoxa13/d13* mutants reveals important roles for this group in autopod patterning (11).

In addition, the *HoxA/D9–13* paralogous group genes are collectively required for the activation and maintenance of *Shh*

expression in limb AP patterning (12, 13). Although misexpression of more anterior *Hox* genes in mice reportedly affects limb patterning (16), no loss-of-function mutants of anterior, non-*abdominal B* (*AbdB*)-related genes have demonstrated defects in the patterning of limb skeletal elements. Moreover, no *HoxB* or *HoxC* group genes had been shown to play a role in forelimb development until a report by our group demonstrated that all four *Hox9* paralogous genes (*Hoxa9*, *Hoxb9*, *Hoxc9*, and *Hoxd9*) are required in the early lateral plate mesoderm to define the posterior forelimb field by regulating the onset of *Hand2* expression (15).

Numerous human syndromes and mouse mutants that affect AP limb patterning have been identified. Disruption of *Shh* expression accounts for some of these phenotypes. Some mutations in the *Shh* limb enhancer ZRS lead to loss of posterior digits reminiscent of loss of *Shh* function (3, 4, 8, 17). In addition, many point mutations in the ZRS identified in spontaneous mouse mutants (*Hx* and *M100081*) (18), human patients (PPD2, Cuban mutation, Werner mesomelic syndrome, and others) (5–7, 18–27), chickens (17), and cats (28) that lead to anteriorized and/or ectopic expression of *Shh*, indicating that the ZRS enhancer not only directs activation of *Shh* in the ZPA, but also is responsible for repression of *Shh* in the anterior limb.

## Significance

Mammalian *Hox* genes are important for limb development. Posterior *abdominal B* (*AbdB*) *Hox* groups (*Hox9–Hox13*) are required for establishment of the limb proximodistal axis. In addition, *Hox9* genes control the onset of *Hand2* expression in the posterior forelimb, and *HoxA/D AbdB* genes are responsible for the initiation and maintenance of *Sonic Hedgehog* (*Shh*). In this study, we generated *Hox5* triple mutants, resulting in embryos with severe forelimb anterior patterning defects. We found that *Hox5* proteins interact with *promyelocytic leukemia zinc finger* to restrict *Shh* expression in the forelimb bud. The hindlimb in *Hox5* mutants develops normally, revealing distinct differences in anteroposterior field establishment in the forelimb and hindlimb and unanticipated roles for non-*AbdB Hox* genes, including *HoxB* and *HoxC* group genes, in limb development.

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Mutations in factors that have not been associated with *Shh* signaling also can lead to anterior limb defects. For example, patients with Holt–Oram syndrome (HOS) and Okhiro syndrome (OS), which are caused by mutations of *TBX5* and the *Spalt* family zinc finger transcription factor *SALL4*, respectively, show anterior forelimb defects, including loss of a thumb or a triphalangeal digit and/or hypoplasia of the radius (29–36). Loss of function of promyelocytic leukemia zinc finger gene (*Plzf*) function in both human patients and mouse mutants also results in similar limb AP patterning defects (37–39). Interactions among the myriad of required factors in limb AP patterning and their relationships to *Shh* signaling and other signaling pathways remain incompletely understood.

In this study, we demonstrate that *Hox5* genes perform a novel function in limb AP patterning. Loss of function of *Hox5* paralogous genes (*Hoxa5*, *Hoxb5*, and *Hoxc5*), an anterior set of *Hox* genes not belonging to the *AbdB*-related *Hox* group, results in defects in anterior forelimb patterning that closely resemble some point mutations in the ZRS in both mice and humans. Early patterning of the anterior and posterior limb compartments is not disrupted in these mutants; however, the limb defects in *Hox5* mutants are associated with ectopic *Shh* expression in the anterior forelimb buds, and we provide molecular and genetic evidence indicating that *Hox5* interacts with *Plzf* to restrict *Shh* expression and pattern the anterior forelimb.

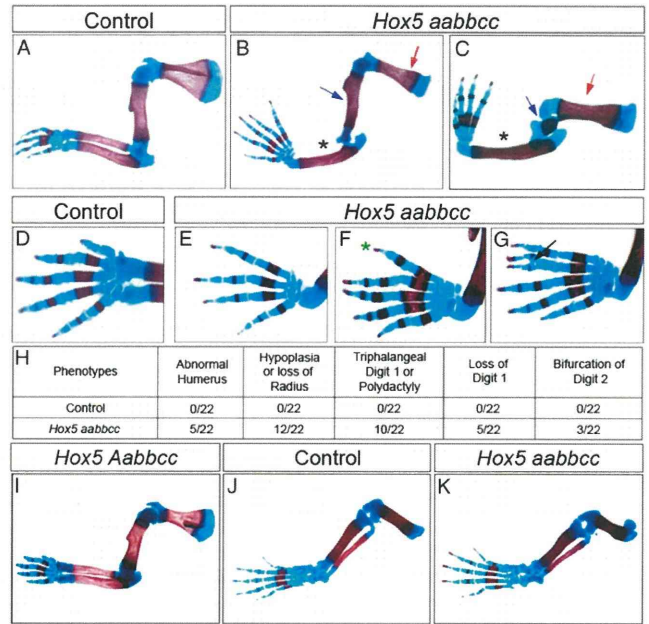
## Results

**Inactivation of *Hox5* Paralogous Group Genes Results in Anterior Forelimb Defects.** Single mutants for *Hoxa5*, *Hoxb5*, and *Hoxc5* (the three mammalian *Hox5* paralogous group genes) have been generated previously (40–42). Although loss of *Hoxa5* function results in a smaller scapula (43), no limb patterning abnormalities have been reported for any of the three *Hox5* single mutants despite the expression of these genes in the developing forelimb and hindlimb (42, 43) (Fig. S1). Compound mutants deficient for any combination of as many as five of the six *Hox5* alleles did not exhibit limb defects (Fig. 1 *A* and *I*). Only when all six *Hox5* alleles were mutated were defects in the anterior forelimb skeletal elements observed (Fig. 1 *A–H*). The humerus of *Hox5* triple mutants was variably affected (Fig. 1 *B* and *C*), the radius was truncated or lost (Fig. 1 *B* and *C*), and digit 1 was often missing or transformed into a triphalangeal digit, with the distal portion of digit 2 occasionally bifurcated (Fig. 1 *E–G*). Hindlimb development was not affected in *Hox5* mutant mice, even though *Hox5* was expressed at early hindlimb bud stages (Fig. 1 *J* and *K*).

***Shh* Is Ectopically Activated in *Hox5* Mutant Forelimb Buds.** Given the clear disruption of AP limb patterning and the importance of *Shh* in this process, we examined *Shh* expression in *Hox5* mutants. *Shh* expression expanded anteriorly in early forelimb buds of *Hox5* mutants, and ectopic *Shh* expression was observed in anterior domains in some instances (Fig. 2 *A–E*). The expression of downstream factors *Ptch1* and *Gli1* was consistently anteriorized in *Hox5* triple-mutant embryos (Fig. 2 *F, G, J*, and *K*). *Fgf4* expression in the AER extended anteriorly compared with controls (Fig. 2 *H* and *L*), consistent with anteriorized *Shh* expression, whereas *Fgf8* was expressed normally in the AER (Fig. 2 *I* and *M*).

Because *Shh* expression is disrupted at early stages, we examined AP patterning regulators upstream of *Shh* signaling. In somite-matched *Hox5* mutants and controls, there were no observable differences in the expression of *Gli3* (Fig. 3 *A, C, F*, and *H*) or *Hand2* (Fig. 3 *B, D, G*, and *I*) at any stage examined. *Alx4*, another early regulator of anterior limb patterning, was expressed normally in *Hox5* mutants (Fig. 3 *E* and *J*).

Misexpression of *HoxD* genes results in preaxial polydactyly phenotypes bearing some resemblance to *Hox5* mutants (12, 13, 16, 44), and ectopic or anteriorized *Shh* expression also leads to coincident anteriorization of *Hoxd10–13* (45). Expression of



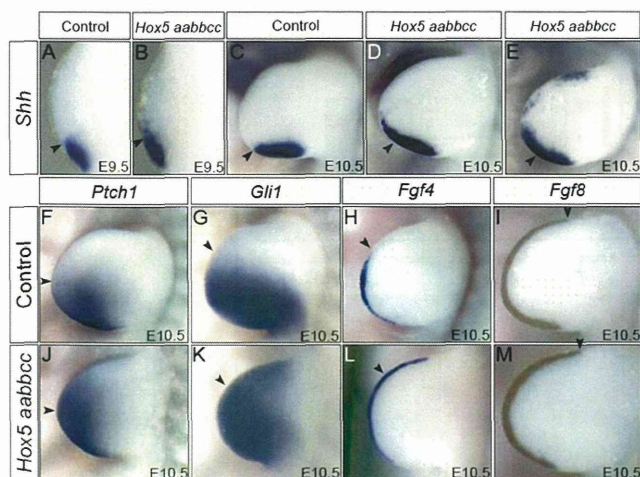
**Fig. 1.** Loss of function of *Hox5* paralogous genes results in anterior forelimb defects. (*A–G*) Skeletal analysis of control and *Hox5* triple-mutant forelimbs at E18.5. The scapula is reduced in *Hox5* triple mutants compared with controls, as observed for *Hoxa5* single mutants (*A–C*, red arrows). The stylopod is reduced or truncated only in embryos with a phenotype in the radius (*B* and *C*, blue arrow). The radius of mutant forelimbs is missing or severely truncated (*B* and *C*, black asterisk). (*D–G*) The most anterior digit develops abnormally in *Hox5* mutants compared with controls. Digit 1 is often missing (*E*) or triphalangeal (*F* and *G*, green asterisk). Less frequently, *Hox5* mutants also have a bifurcated digit 2 (*G*, black arrow). Digit phenotypes do not correlate with the severity of stylopod/zeugopod defects. (*H*) Table summarizing forelimb phenotypes of *Hox5* mutant forelimbs. (*I*) Compound mutants deficient for as many as five of the six *Hox5* alleles do not exhibit limb defects. (*J* and *K*) Hindlimb development is not affected in *Hox5* mutants.

*Hoxd10–13* genes in *Hox5* mutants was shifted anteriorly in *Hox5* mutant forelimbs at embryonic day (E) 10.5 (Fig. 3 *K–R*), consistent with misregulation of *Shh*. It is important to note that *HoxD* genes are not linked to the *HoxA*, *HoxB*, or *HoxC* clusters, and thus these effects cannot be due to *cis* effects from the targeted mutations introduced into the *Hox5* alleles.

***Plzf* Is a Potential Coregulator of *Shh* Repression.** Several additional regulators of anterior limb patterning have been identified in human disease syndromes as well as in mouse mutants. Forelimb defects similar to those seen in *Hox5* mutants have been identified in patients with HOS caused by *Tbx5* mutations (32–36), and *Hox* genes have been reported to be capable of driving *Tbx5* expression (46), OS caused by *Sall4* mutations (29, 30), Townes–Brooks syndrome caused by *Sall1* mutations (31, 47) and Saethre–Chotzen syndrome caused by *Twist1* mutations, which also show limb phenotypes in mutant mice (48–50). Mutations of both the human and mouse limb enhancer of *Shh*, ZRS (7, 18, 19, 24), and human multiple congenital anomaly/mental retardation syndromes caused by mutations of *Plzf*, as well as loss-of-function mutations in *Plzf* in mice (37, 39), lead to similar phenotypes. Based on these similarities, we investigated the expression of *Tbx5*, *Sall1*, *Sall4*, *Twist1*, and *Plzf* in our *Hox5* mutants. We found no change in the expression of any of these genes in *Hox5* mutant forelimbs (Figs. S2 *A–L* and S3 *A–D*).

To test whether these factors might act in parallel in the same pathway as *Hox5* and affect *Shh* expression, we examined *Shh*





**Fig. 2.** *Shh* signaling is disrupted in *Hox5* mutant forelimbs. (A–E) *Shh* expression is anteriorized in *Hox5* mutant forelimbs. At E9.5, *Hox5* mutant forelimbs display slightly anteriorized *Shh* expression compared with controls (A and B). Anteriorization of *Shh* expression is observed by E10.5 in *Hox5* mutant forelimbs compared with controls (C–E), and ectopic *Shh* expression appears in anterior regions of some mutant forelimbs (E). (F–M) Expression of *Ptch1* and *Gli1* is consistently shifted anteriorly in *Hox5* mutants at E10.5 compared with controls (F, J, G, and K). *Fgf4* expression is also anteriorized in *Hox5* mutant forelimbs compared with controls (H and L), whereas *Fgf8* expression is unchanged (I and M). Black arrowheads in each panel mark the WT anterior boundary of expression for each probe.

expression in *Tbx5* heterozygotes with or without loss of *Sall4* function, as well as in *Plzf* mutant embryos. Consistent with previous reports that *Shh* expression is not altered with loss of *Tbx5* function (51), we found that *Shh* was not altered in *Tbx5* or *Sall4* heterozygous mutants or compound *Tbx5/Sall4* heterozygous mutants (Fig. S3 I–L); however, *Plzf* mutants showed a small but reproducible anterior shift in *Shh* expression (Fig. 4 A and B). In addition, *Shh* transcripts were increased by ~50% in *Plzf* mutant limbs (Fig. 4C). To confirm changes in *Shh* expression, we examined the expression of *Ptch1* and *Gli1*, factors immediately downstream of *Shh*. In *Plzf* mutants, *Ptch1* and *Gli1* were consistently anteriorized and ectopically expressed (Fig. 4 D–G), demonstrating that *Shh* expression is affected downstream of *Plzf* in the developing limb.

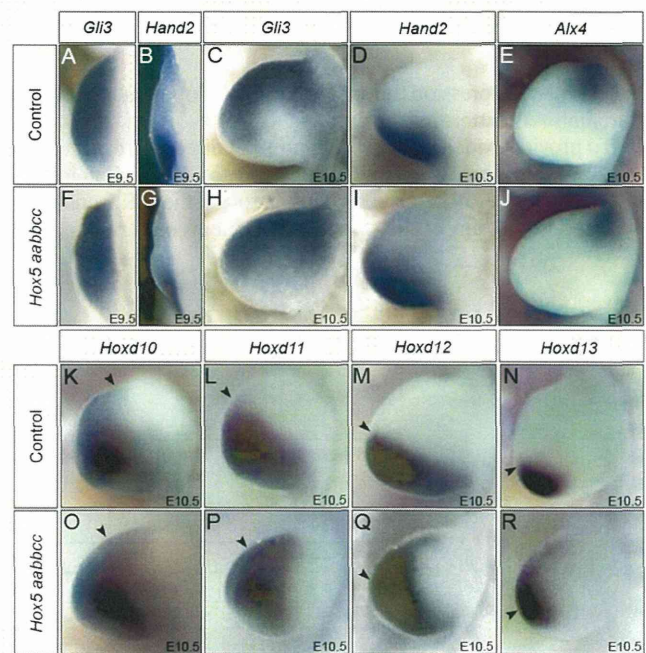
**Hox5 Proteins Interact with *Plzf* and Can Regulate *Shh* Expression Through the ZRS in Vitro.** *Plzf* mouse mutants have been found to have similar forelimb phenotypes as *Hox5* mutant mice, although with low penetrance (37). The mice used in the present study demonstrated a similar phenotype (Fig. 5 A and B), but with 100% penetrance in the forelimb (52). Heterozygous embryos had no limb phenotype (Fig. 5C). Having already demonstrated no changes in *Plzf* expression in our *Hox5* mutants, we investigated the possibility that *Hox5* acts downstream of *Plzf* in forelimb AP patterning, but found normal *Hox5* expression levels in *Plzf* mutant forelimbs (Fig. S3 E–G).

To test whether *Hox5* and *Plzf* proteins are capable of interacting to regulate downstream limb target genes, we examined potential physical interactions between these proteins in vitro. In cells cotransfected with epitope-tagged *Hoxa5*, *Hoxb5*, or *Hoxc5* protein in combination with tagged *Plzf*, we found coprecipitation of all three *Hox5* proteins with *Plzf* protein (Fig. 4H and Fig. S3H), consistent with the possibility that these proteins interact to regulate downstream targets.

If *Hox5* and *Plzf* function together to repress *Shh* expression anteriorly and affect AP patterning of the forelimb, then we would expect these genes to interact in vivo. *Hox5* mutants

demonstrate no forelimb defects unless all six alleles of the three *Hox5* genes are mutated (compare Fig. 1 A–G and Fig. 5D). *Plzf* mutants exhibited forelimb phenotypes only with loss of both alleles, whereas heterozygous animals had no phenotype (Fig. 5 A–C). Compound mutants heterozygous for *Plzf* combined with either one or two mutant *Hox5* alleles did not exhibit any forelimb defects. Embryos heterozygous for *Plzf* plus three *Hox5* mutant alleles resulted in preaxial limb skeletal defects in only 1 of 18 forelimbs (Fig. 5G). Compound mutants heterozygous for *Plzf* plus four *Hox5* mutant alleles showed more severe forelimb defects with higher penetrance (Fig. 5 E and G). Forelimb defects were further exacerbated in compound mutants heterozygous for *Plzf* plus five *Hox5* mutant alleles, with 12 of 14 forelimbs demonstrating anterior forelimb defects (Fig. 5 F and G). These findings indicate that strong genetic interactions occur between *Hox5* and *Plzf* in vivo, supporting the hypothesis that these proteins cooperatively regulate forelimb AP patterning events.

If *Hox5* and *Plzf* coordinately regulate *Shh* expression, then the phenotypes observed in the compound mutants should result in changes in *Shh* pathway expression. Obvious anteriorization of *Shh* expression was observed in *Plzf/Hox5* compound mutants (Fig. 5 H and I), similar to that seen in both *Hox5* triple mutants and *Plzf* mutants, but not in *Plzf* heterozygotes or in compound *Hox5* mutants carrying up to five mutant alleles. *Gli1* and *Ptch1* expression also was anteriorized in the forelimb of the compound *Hox5/Plzf* mutants (Fig. 5 J–M), but not in control WT embryos, *Plzf* heterozygotes, or compound *Hox5* mutants harboring up to five mutant alleles. These genetic data further support the assertion that *Hox5* and *Plzf* cooperatively repress



**Fig. 3.** Early limb patterning pathways are not disrupted, but posterior *HoxD* gene expression is anteriorized in *Hox5* mutants. (A–J) Early AP patterning factors are not disrupted in *Hox5* mutants. The expression of *Gli3* is not altered at E9.5 (A and F) or E10.5 (C and H). *Hand2* is expressed normally in *Hox5* mutant forelimbs at E9.5 (B and G) and E10.5 (D and I). *Alx4* expression in *Hox5* mutants is also comparable to that in controls at E10.5 (E and J). (K–R) The expression limits of posterior *HoxD* genes are anteriorized in E10.5 *Hox5* mutant forelimbs. *Hoxd10* (K and O), *Hoxd11* (L and P), *Hoxd12* (M and Q), and *Hoxd13* (N and R) are all expressed more anteriorly in *Hox5* mutant forelimb buds (O–R) compared with controls (K–N). Black arrowheads mark the WT anterior boundary of expression for each probe.







attempts to perform ChIP of both *Plzf* and *Hox5* at the ZRS promoter *in vivo*. Despite the successful use of the antibodies at other enhancers, the very high level of background (control antibody) binding at the ZRS and the potentially small numbers of responding cells in the limb bud precluded conclusive evidence of binding of these proteins *in vivo* (Fig. S4 and *SI Materials and Methods*).

## Discussion

In this study, we show a unique and unexpected role for *Hox5* genes in limb AP patterning. A myriad of genetic studies have defined important roles for *HoxA* and *HoxD* group 9–13 genes in forelimb development (9–14). Recently, we reported that *Hox9* genes from *HoxB* and *HoxC* complex, along with *Hoxa9* and *Hoxd9*, are also required to define the posterior forelimb compartment (15). However, previous loss-of-function studies have provided no evidence that anterior, non-*AbdB Hox* genes participate in patterning limb skeletal elements. Here we report limb phenotypes resulting from loss of *Hox5* paralogous gene function, further demonstrating pivotal roles for *HoxB* and *HoxC* complex genes in forelimb AP patterning, with *Hoxa5*, *Hoxb5*, and *Hoxc5* controlling anterior forelimb patterning.

The limb defects in our *Hox5* mutants are restricted to forelimbs, with no defects in hindlimb development observed. The limb defects in quadruple *Hox9* mutants are also restricted to the forelimbs (15). Taken together, our findings highlight significant differences in how anterior and posterior limb compartments are established in forelimbs and hindlimbs. This is surprising, considering the downstream factors currently known to play critical roles in AP patterning (i.e., *Hand2*, *Gli3*, and *Shh*) function similarly in both forelimbs and hindlimbs. Our findings from both loss of *Hox9* paralog function (15) and the present study suggest that early axial *Hox* expression in the lateral plate mesoderm controls the establishment of the anterior (*Hox5*) and posterior (*Hox9*) compartments of the forelimb. None of the numerous combinations of posterior *Hox* loss-of-function mutants reported to date (9–14) are known to lead to hindlimb AP patterning defects analogous to those reported for loss of *Hox9* or *Hox5* paralogs. Itou et al. (53) recently demonstrated that LIM-homeodomain factor *Islet1* is a critical regulator of *Hand2* expression and the posterior compartment in hindlimbs. How the hindlimb anterior compartment is established remains to be discovered.

It is also interesting to note that although *Hox5* paralogs and *Hox9* paralogs control anterior and posterior patterning, respectively, *Hox9* paralogs are responsible for initiation of *Hand2*, whereas no disruption of early anterior/posterior compartment formation is observed in *Hox5* mutants (ref. 15 and this paper). In *Hox5* mutant limbs, the initial *Hand2/Gli3* pattern is normal, but downstream expression of *Shh* is affected, consistent with *Hox5* regulating *Shh* expression more directly. This finding is also consistent with previous reports demonstrating that numerous point mutations in the ZRS of mouse, humans, chickens, and cats result in ectopic *Shh* activity in anterior domains of the limb bud and result in phenotypes similar to those that we detected in *Hox5* mutant mice, including defects in the stylopod, anterior zeugopod, and digits (5–7, 17–28).

The findings reported here also reveal a role for *Plzf* in regulating *Shh* expression in limb AP patterning. We detected anteriorized *Shh* expression in *Plzf* mutants, in contrast to a previous report (37). The discrepancy may be related to the use of different *Plzf* mutant alleles; we observed 100% penetrance of forelimb defects in the mutants used in the present study, sig-

nificantly higher than in the previously reported mutant allele (37). Our finding that *Shh* is regulated downstream of *Plzf* in limb AP patterning is further supported by changes in *Ptch1* and *Gli1* expression in addition to *Shh* expression.

Our genetic and molecular analyses of *Hox5* function in forelimb development support a model in which *Hox5* proteins, interacting with *Plzf*, act as repressors of *Shh* expression. Among the many potential binding sites in the ZRS are several putative *Hox*-binding sites and at least one putative *Plzf*-binding site. The putative *Plzf* site is mutated in the “Cuban mutation,” one of the human mutations with limb phenotypes similar to those in the mice reported here, including radial aplasia (7). There is also a report of three independent probands with anterior forelimb phenotypes (mostly triphalangeal thumbs) that harbor a mutation in one of the three putative *Hox*-binding sites (19).

The activity of *Hox5* and *Plzf* likely combine with numerous other factors that bind to this enhancer to both activate and repress *Shh* expression. Several factors, including *Hand2* and *HoxD*, have been shown to activate *Shh* expression via the ZRS limb enhancer element (54, 55). *Etv4/Etv5* and *Twist 1* have been shown to cooperate to restrict *Shh* expression to the posterior limb bud (50, 56). The ZRS is more than 700 bp long, and thus it is likely that a myriad of factors converge at this critical regulatory hub to direct proper expression of *Shh* in the developing vertebrate limb. A complete understanding of the factors that bind to these sites and how they interact remains to be delineated in future studies.

## Materials and Methods

**Mice and Whole-Mount *In Situ* Hybridization.** All mouse mutant strains used in this study have been reported previously (41, 52). Control mice included both WT embryos and low-allele littermates from *Hox* and *Hox/Plzf* crosses. The results were identical, and thus we use the term “control” throughout for clarity. Mutant mouse strains, early skeletal preparations, and standard whole-mount *in situ* hybridization were as described previously (14, 41, 52). All *in situ* probes were prepared as described previously (15, 57, 58). All experiments were performed following protocols approved by the University of Michigan’s Institutional Committee on the Use and Care of Animals.

**Cell Culture, Transfections, Luciferase Assays, and Coimmunoprecipitation Assays.** HEK293 or HEK293T cells were used and plated as described previously (59). Cell transfections were performed by CaPO<sub>4</sub> precipitation. Coimmunoprecipitation assays were performed as described previously (59). *Hox5* and *Plzf* protein-coding sequences were amplified from their cDNAs using the primers listed in *SI Materials and Methods*, then subcloned into pCS2+MT or p3XFlag-CMV vectors (Sigma-Aldrich). Details of plasmid generation and reporter assays have been reported previously (59). The highly conserved ZRS core region was amplified using the primers listed in *SI Materials and Methods* and then subcloned into a pGL3 promoter vector (Promega). The Student *t* test was used to determine statistical significance. All experiments were repeated at least three times in independent experiments.

**RNA Isolation and Quantitative RT-PCR.** RNA was isolated from mouse limbs with the Qiagen RNeasy Micro Kit. Quantitative RT-PCR (qRT-PCR) was carried out using Roche FastStart SYBR Green Master Mix. Primer sequences have been described previously (51). Relative expression values were calculated as 2<sup>-ΔΔC<sub>t</sub></sup>, and values of controls were normalized to 1. GAPDH served as an internal control for normalization in all qRT-PCR experiments, and the Student *t* test was used to determine statistical significance (*P* < 0.05). All experiments were repeated at least three times.

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1. Towers M, Tickle C (2009) Generation of pattern and form in the developing limb. *Int J Dev Biol* 53(5–6):805–812.
2. Riddle RD, Johnson RL, Laufer E, Tabin C (1993) Sonic hedgehog mediates the polarizing activity of the ZPA. *Cell* 75(7):1401–1416.

3. Chiang C, et al. (2001) Manifestation of the limb prepattern: Limb development in the absence of sonic hedgehog function. *Dev Biol* 236(2):421–435.
4. Kraus P, Fraidtenraich D, Loomis CA (2001) Some distal limb structures develop in mice lacking Sonic hedgehog signaling. *Mech Dev* 100(1):45–58.



5. Lettice LA, et al. (2003) A long-range Shh enhancer regulates expression in the developing limb and fin and is associated with preaxial polydactyly. *Hum Mol Genet* 12(14):1725–1735.
6. Wiczkorek D, Köster B, Gillessen-Kaesbach G (2002) Absence of thumbs, A/hypoplasia of radius, hypoplasia of ulnae, retarded bone age, short stature, microcephaly, hypoplastic genitalia, and mental retardation. *Am J Med Genet* 108(3):209–213.
7. Zguricas J, et al. (1999) Clinical and genetic studies on 12 preaxial polydactyly families and refinement of the localisation of the gene responsible to a 1.9 cM region on chromosome 7q36. *J Med Genet* 36(1):32–40.
8. Sagai T, Hosoya M, Mizushima Y, Tamura M, Shiroishi T (2005) Elimination of a long-range cis-regulatory module causes complete loss of limb-specific Shh expression and truncation of the mouse limb. *Development* 132(4):797–803.
9. Davis AP, Witte DP, Hsieh-Li HM, Potter SS, Capecchi MR (1995) Absence of radius and ulna in mice lacking *hoxa-11* and *hoxd-11*. *Nature* 375(6534):791–795.
10. Fromental-Ramain C, et al. (1996) Specific and redundant functions of the paralogous *Hoxa-9* and *Hoxd-9* genes in forelimb and axial skeleton patterning. *Development* 122(2):461–472.
11. Fromental-Ramain C, et al. (1996) *Hoxa-13* and *Hoxd-13* play a crucial role in the patterning of the limb autopod. *Development* 122(10):2997–3011.
12. Kmita M, et al. (2005) Early developmental arrest of mammalian limbs lacking *HoxA/HoxD* gene function. *Nature* 435(7045):1113–1116.
13. Tarchini B, Duboule D, Kmita M (2006) Regulatory constraints in the evolution of the tetrapod limb anterior-posterior polarity. *Nature* 443(7114):985–988.
14. Wellik DM, Capecchi MR (2003) *Hox10* and *Hox11* genes are required to globally pattern the mammalian skeleton. *Science* 301(5631):363–367.
15. Xu B, Wellik DM (2011) Axial *Hox9* activity establishes the posterior field in the developing forelimb. *Proc Natl Acad Sci USA* 108(12):4888–4891.
16. Knezevic V, et al. (1997) *Hoxd-12* differentially affects preaxial and postaxial chondrogenic branches in the limb and regulates Sonic hedgehog in a positive feedback loop. *Development* 124(22):4523–4536.
17. Maas SA, Suzuki T, Fallon JF (2011) Identification of spontaneous mutations within the long-range limb-specific Sonic hedgehog enhancer (ZRS) that alter Sonic hedgehog expression in the chicken limb mutants oligozeugodactyly and silkie breed. *Dev Dyn* 240(5):1212–1222.
18. Sagai T, et al. (2004) Phylogenetic conservation of a limb-specific, cis-acting regulator of Sonic hedgehog (Shh). *Mamm Genome* 15(1):23–34.
19. Furniss D, et al. (2008) A variant in the sonic hedgehog regulatory sequence (ZRS) is associated with triphalangeal thumb and deregulates expression in the developing limb. *Hum Mol Genet* 17(16):2417–2423.
20. Albuissou J, et al. (2011) Identification of two novel mutations in Shh long-range regulator associated with familial pre-axial polydactyly. *Clin Genet* 79(4):371–377.
21. Farooq M, et al. (2010) Preaxial polydactyly/triphalangeal thumb is associated with changed transcription factor-binding affinity in a family with a novel point mutation in the long-range cis-regulatory element ZRS. *Eur J Hum Genet* 18(6):733–736.
22. Gurnett CA, et al. (2007) Two novel point mutations in the long-range SHH enhancer in three families with triphalangeal thumb and preaxial polydactyly. *Am J Med Genet A* 143(1):27–32.
23. Semerci CN, et al. (2009) Homozygous feature of isolated triphalangeal thumb-preaxial polydactyly linked to 7q36: No phenotypic difference between homozygotes and heterozygotes. *Clin Genet* 76(1):85–90.
24. Wiczkorek D, et al. (2010) A specific mutation in the distant sonic hedgehog (SHH) cis-regulator (ZRS) causes Werner mesomelic syndrome (WMS), while complete ZRS duplications underlie Haas type polysyndactyly and preaxial polydactyly (PPD) with or without triphalangeal thumb. *Hum Mutat* 31(1):81–89.
25. Wu L, et al. (2009) A ZRS duplication causes syndactyly type IV with tibial hypoplasia. *Am J Med Genet A* 149A(4):816–818.
26. Laurell T, et al. (2012) A novel 13 base pair insertion in the sonic hedgehog ZRS limb enhancer (ZRS/LMBR1) causes preaxial polydactyly with triphalangeal thumb. *Hum Mutat* 33(7):1063–1066.
27. Al-Qattan MM, Al Abdulkareem I, Al Haidan Y, Al Balwi M (2012) A novel mutation in the SHH long-range regulator (ZRS) is associated with preaxial polydactyly, triphalangeal thumb, and severe radial ray deficiency. *Am J Med Genet A* 158A(10):2610–2615.
28. Lettice LA, Hill AE, Devenney PS, Hill RE (2008) Point mutations in a distant sonic hedgehog cis-regulator generate a variable regulatory output responsible for preaxial polydactyly. *Hum Mol Genet* 17(7):978–985.
29. Al-Baradie R, et al. (2002) Duane radial ray syndrome (Okhiro syndrome) maps to 20q13 and results from mutations in SALL4, a new member of the SAL family. *Am J Hum Genet* 71(5):1195–1199.
30. Kohlhasse J, et al. (2002) Okhiro syndrome is caused by SALL4 mutations. *Hum Mol Genet* 11(23):2979–2987.
31. Kohlhasse J, Wischermann A, Reichenbach H, Froster U, Engel W (1998) Mutations in the SALL1 putative transcription factor gene cause Townes-Brocks syndrome. *Nat Genet* 18(1):81–83.
32. Basson CT, et al. (1997) Mutations in human TBX5 [corrected] cause limb and cardiac malformation in Holt-Oram syndrome. *Nat Genet* 15(1):30–35.
33. Basson CT, et al. (1999) Different TBX5 interactions in heart and limb defined by Holt-Oram syndrome mutations. *Proc Natl Acad Sci USA* 96(6):2919–2924.
34. Brassington AM, et al. (2003) Expressivity of Holt-Oram syndrome is not predicted by TBX5 genotype. *Am J Hum Genet* 73(1):74–85.
35. Fan C, Liu M, Wang Q (2003) Functional analysis of TBX5 missense mutations associated with Holt-Oram syndrome. *J Biol Chem* 278(10):8780–8785.
36. Li QY, et al. (1997) Holt-Oram syndrome is caused by mutations in TBX5, a member of the Brachyury (T) gene family. *Nat Genet* 15(1):21–29.
37. Barna M, Hawe N, Niswander L, Pandolfi PP (2000) Plzf regulates limb and axial skeletal patterning. *Nat Genet* 25(2):166–172.
38. Barna M, et al. (2002) Plzf mediates transcriptional repression of HoxD gene expression through chromatin remodeling. *Dev Cell* 3(4):499–510.
39. Fischer S, et al. (2008) Biallelic loss of function of the promyelocytic leukaemia zinc finger (PLZF) gene causes severe skeletal defects and genital hypoplasia. *J Med Genet* 45(11):731–737.
40. Jeannotte L, Lemieux M, Charron J, Poirier F, Robertson EJ (1993) Specification of axial identity in the mouse: Role of the *Hoxa-5* (*Hox1.3*) gene. *Genes Dev* 7(11):2085–2096.
41. McIntyre DC, et al. (2007) Hox patterning of the vertebrate rib cage. *Development* 134(16):2981–2989.
42. Rancourt DE, Tsuzuki T, Capecchi MR (1995) Genetic interaction between *hoxb-5* and *hoxb-6* is revealed by nonallelic noncomplementation. *Genes Dev* 9(1):108–122.
43. Aubin J, Déry U, Lemieux M, Chailier P, Jeannotte L (2002) Stomach regional specification requires *Hoxa5*-driven mesodermal-epithelial signaling. *Development* 129(17):4075–4087.
44. Zakany J, Zacchetti G, Duboule D (2007) Interactions between HOXD and Gli3 genes control the limb apical ectodermal ridge via Fgf10. *Dev Biol* 306(2):883–893.
45. Wada N, Kawakami Y, Nohno T (1999) Sonic hedgehog signaling during digit pattern duplication after application of recombinant protein and expressing cells. *Dev Growth Differ* 41(5):567–574.
46. Minguillon C, et al. (2012) Hox genes regulate the onset of Tbx5 expression in the forelimb. *Development* 139(17):3180–3188.
47. Kohlhasse J, et al. (1999) Molecular analysis of SALL1 mutations in Townes-Brocks syndrome. *Am J Hum Genet* 64(2):435–445.
48. Bourgeois P, et al. (1998) The variable expressivity and incomplete penetrance of the twist-null heterozygous mouse phenotype resemble those of human Saethre-Chotzen syndrome. *Hum Mol Genet* 7(6):945–957.
49. Krawchuk D, et al. (2010) Twist1 activity thresholds define multiple functions in limb development. *Dev Biol* 347(1):133–146.
50. Zhang Z, et al. (2010) Preaxial polydactyly: interactions among ETV, TWIST1 and HAND2 control anterior-posterior patterning of the limb. *Development* 137(20):3417–3426.
51. Hasson P, Del Buono J, Logan MP (2007) Tbx5 is dispensable for forelimb outgrowth. *Development* 134(1):85–92.
52. Buas FW, et al. (2004) Plzf is required in adult male germ cells for stem cell self-renewal. *Nat Genet* 36(6):647–652.
53. Itou J, et al. (2012) *Isl1* regulates establishment of the posterior hindlimb field upstream of the Hand2-Shh morphoregulatory gene network in mouse embryos. *Development* 139(9):1620–1629.
54. Capellini TD, et al. (2006) Pbx1/Pbx2 requirement for distal limb patterning is mediated by the hierarchical control of Hox gene spatial distribution and Shh expression. *Development* 133(11):2263–2273.
55. Galli A, et al. (2010) Distinct roles of Hand2 in initiating polarity and posterior Shh expression during the onset of mouse limb bud development. *PLoS Genet* 6(4):e1000901.
56. Zhang Z, Verheyden JM, Hassell JA, Sun X (2009) FGF-regulated ETV genes are essential for repressing Shh expression in mouse limb buds. *Dev Cell* 16(4):607–613.
57. Verheyden JM, Lewandoski M, Deng C, Harfe BD, Sun X (2005) Conditional inactivation of *Fgfr1* in mouse defines its role in limb bud establishment, outgrowth and digit patterning. *Development* 132(19):4235–4245.
58. Avantsaggiato V, et al. (1995) Developmental analysis of murine Promyelocyte Leukemia Zinc Finger (PLZF) gene expression: Implications for the neuromeric model of the forebrain organization. *J Neurosci* 15(7 Pt 1):4927–4942.
59. Gong KQ, Yallowitz AR, Sun H, Dressler GR, Wellik DM (2007) A Hox-Eya-Pax complex regulates early kidney developmental gene expression. *Mol Cell Biol* 27(21):7661–7668.



# Epigenetic factors and cardiac development

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## Abstract

Congenital heart malformations remain the leading cause of death related to birth defects. Recent advances in developmental and regenerative cardiology have shed light on a mechanistic understanding of heart development that is controlled by a transcriptional network of genetic and epigenetic factors. This article reviews the roles of chromatin remodelling factors important for cardiac development with the current knowledge of cardiac morphogenesis, regeneration, and direct cardiac differentiation. In the last 5 years, critical roles of epigenetic factors have been revealed in the cardiac research field.

## Keywords

Epigenetics • Chromatin remodelling factors • Baf60c • Cardiac transcription factors • Cardiac cell fate • Cardiac reprogramming

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## 1. Introduction: cardiac morphogenesis and diseases

The heart is the first functional organ to form in developing embryos, and cardiogenesis takes place in a highly conserved manner from insects to vertebrates. After the formation of three germ layers (ectoderm, endoderm, and mesoderm), pre-cardiac mesodermal cells arise bilaterally from the nascent mesoderm. These cells migrate into the midline and differentiate, giving rise to the contractile heart.

In mammals and birds, the bilateral cardiogenic mesodermal cells migrate and merge at the anterior midline to generate the cardiac crescent, a crescent-shaped heart-forming region at the cranial border of the embryonic disc.<sup>1–3</sup> Upon folding of the embryonic disc, the cardiac crescent positions towards a developing neck area of the embryo and the edges of the cardiac crescent migrate to fuse and form the primitive heart tube.<sup>4</sup> The resulting heart tube undergoes the process of looping and chamber formation, accompanied by the activation of specific cardiac gene expression programmes required for the differentiation and maturation of pre-cardiac cells to generate the myocardium of the atrial and ventricular chambers, as well as the inflow tract, atrioventricular canal, and outflow tract.<sup>5,6</sup>

Two distinct pools of pre-cardiac fields were identified, both of which contribute to heart formation.<sup>7</sup> The first heart field (FHF) cells are derived from the cardiac crescent and give rise to the

formation of the left ventricle and a part of the atria.<sup>8</sup> Cells from the second heart field (SHF), described by Kelly *et al.*,<sup>9</sup> are located in the pharyngeal mesoderm dorsal to the heart tube, giving rise to the outflow tract and right ventricle myocardium at the arterial pole of the heart.<sup>9–11</sup>

The induction, expansion, and differentiation of pre-cardiac cells are controlled by various signalling molecules, including bone morphogenetic proteins (BMPs),<sup>12</sup> fibroblast growth factors (FGFs),<sup>8</sup> and wingless-related MMTV integration site (Wnt) proteins.<sup>13</sup> BMP and FGF signals are important for the induction and differentiation of cardiogenesis.<sup>12,14,15</sup> BMPs play a key role in the specification of FHF cells by activating the expression of cardiac transcription factors such as Nkx2–5, Gata4, and Tbx5.<sup>16,17</sup> SHF progenitors, on the other hand, require Wnt/ $\beta$ -catenin signalling for their proper development.<sup>13,18,19</sup> Wnt/ $\beta$ -catenin signals positively regulate the expansion of SHF progenitors and affect the expression of *Islet1* (*Isl1*), a marker for multi-potent cardiac progenitor cells (CPCs).<sup>18,20,21</sup>

The mammalian heart consists of various cell types, including atrial and ventricular cardiomyocytes, fibroblasts, endocardial cells, epicardial cells, cells from the conduction system (sinoatrial node, atrioventricular node, purkinje fibers), smooth muscle cells making up the aorta and (coronary) arteries, and cells from the autonomous nervous system. Formation of the functional heart requires proper development of these cardiac cells through tight transcriptional regulation of cardiac genes. The fact that congenital cardiac anomalies occur at a high

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